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Automated multi-dimensional liquid chromatography: sample preparation and identification of peptides from human blood filtrate

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Abstract

A comprehensive on-line sample clean-up with an integrated two-dimensional HPLC system was developed for the analysis of natural peptides. Samples comprised of endogenous peptides with molecular weights up to 20 kDa were generated from human hemofiltrate (HF) obtained from patients with chronic renal failure. The (poly-)peptides were separated using novel silica-based restricted access materials with strong cation-exchange functionalities (SCX-RAM). The size-selective sample fractionation step is followed by cation-exchange chromatography as the first dimension. The subsequent second dimension of separation is based on hydrophobic interaction using four parallel short reversed-phase (RP) columns implemented via a fully automated column switching technique. More than 1000 peaks were resolved within the total analysis time of 96 min. Substances of selected peaks were sampled to analyse their molecular weights by off-line MALDI-TOF mass spectrometry and to determine their amino acid sequence by Edman degradation. The potential for comprehensive peptide mapping and identification is demonstrated.

Keywords: Sample preparation; Identification; Human blood filtrate; Peptides

1. Introduction

A study of protein expression in the broadest sense is described as proteomics. Complex human protein mixtures representing parts of the human proteome may be comprised of as many as 100,000 proteins. Proteomics is a research area with fast improvements in methodology as well as new technology introductions [1]. Protein samples of biological origin are by nature highly complex and require sophisticated analytical tools to provide reliable analysis of the components especially in terms of robust, automated and sensitive high-throughput technologies. Most proteomics projects to date involve three distinct steps: sample preparation, separation, and identification.

1.1. Sample preparation by restricted access material (RAM)

Sample preparation is one of the most important and crucial steps in biochemical analysis, and usually the most labour-intensive. Often it represents the slowest and the most cost intensive part of the analytical process, seldomly amenable to automation. The technique of column-switching could avoid these disadvantages. Recently, the most attractive use of column-switching modes utilises restricted access materials. RAMs, which have been introduced by Hagestam and Pinkerton [2] in 1985 and improved by Boos and Grimm [3], combine two chromatographic separation modes in one column, namely size exclusion and adsorption

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chromatography. Owing to the physical and chemical properties of the RAM, macromolecules are excluded and separated rapidly from smaller molecules, enabling the direct processing of biological samples without a protein precipitation step [4]. RAM columns allow direct and repetitive injection as well as size-selective fractionation of untreated biofluids like plasma, serum, urine, fermentation broth and supernatant of tissue homogenates. Besides a defined pore size, the specific feature of RAM is the topochemical bifunctional surface of the particles. At the outer surface, electroneutral diol-groups are bound and exposed to the mobile phase. This layer hampers interactions between the particle and the proteins or other macromolecules that are present in the sample matrix. The inner surface of the porous particle is functionalised and carries anionic (sulfonic acid) groups. It has been recently demonstrated that RAM shows high potential as precolumn sorbents to be used on-line with HPLC for sample fractionation and extraction of peptides [5].

1.2. Separation technologies

Single-dimension separations lack sufficient resolution capability to resolve complex biological matrices or cellular extracts. Giddings [6] demonstrated that the overall peak capacity of multi-dimensional separations is the product of the peak capacities of each independent dimension, but the separation dimensions need to be orthogonal and all components separated in one dimension must remain separated in any additional separation dimension. Separations employing multiple dimensions offer better prospects for proteomics applications. The most efficient high-resolution separation technique available today for protein separation is two-dimensional gel electrophoresis, which was introduced by O'Farrell [7]. However, multi-dimensional chromatography-based technologies coupled to mass spectrometry (MS) are currently being pursued and developed as a complement to 2D-gel electrophoresis [8]. To achieve the criterion of orthogonality, column chromatography with independent stationary phases has been coupled together in different system combinations. Any off-line system that operates by reinjecting the fractions onto a second column is sensitive to loss of sample, vial contamination, sample dilution and low reproducibility. The coupled column system developed by Wagner et al. [9] avoids any fraction storage and automatically performs sample enrichment and desalting, thus avoiding eluent incompatibilities. However, many off-line multi-dimensional HPLC approaches have been described throughout the literature for reasons of technical complexity [10–14]. On the other hand, even off-line systems also often suffer from complexity or lack of automation (e.g. sample loops, fraction collectors, splits and fraction transfers). Simplification and standardisation, based on innovation in both hardware and software, are prerequisites for the creation of automated, robust, and user-friendly proteomics platforms.

1.3. Separation by MD-HPLC combined with mass spectrometry

Chromatography has traditionally played an important role in the analysis of peptides. Chromatography reduces the sample complexity and, thus, supports the identification of especially low-abundant peptides.

Multi-step preseparation methods often are necessary prior to the final chromatographic determination. The column switching technology transforms these multi-step methods into a single-step on-line purification procedure. These systems allow firstly the injection of a large volume of sample to improve the limit of detection and secondly the optimisation of the separation by gradual adjustment of the resolution parameters [15]. Especially in combination with mass spectrometry, the possibility for highly selective substance detection and identification grew dramatically. Wolters et al. [16] introduced an automated method for protein and peptide analysis named multi-dimensional protein identification technology. This technique combines multi-dimensional liquid chromatography with electrospray ionisation tandem mass spectrometry allowing highly sensitive detection of a protein at 100 copies per cell in the background of protein at 1,000,000 copies per cell. Link et al. [17] used and automated the 2D-HPLC method to separate a complex mixture of proteins after tryptic cleavage. Discrete fractions of a strong cation-exchange chromatography were directed to reversed phase columns connected to a mass spectrometer. Opiteck et al. [18] described a 2D-liquid chromatography system which used size-exclusion liquid chromatography followed by RP-LC to separate a mixture of proteins resulting from the lysis of *E. coli* cells detected by MALDI-TOF or ESI mass spectrometry. Several other related approaches of separations have been described recently [10,19,20].

1.4. Peptides from human hemofiltrate

In previous studies, it was shown that peptide hormones are present in human hemofiltrate (HF) in plasma-like concentrations and in their bioactive form [21–23].

HF generated from patients with chronic renal failure has often been used successfully for the isolation of circulating regulatory peptides. Hemofiltration reduces the plasma protein content from 70 g/l in plasma to 50–70 mg/l in HF [24]. The remaining peptide-rich hemofiltrate is extracted by established chromatographic methods as described elsewhere [25]. In brief, 10,000 l HF are separated into nearly 600 chromatographic fractions in a standardised two-step procedure of cation-exchange and reversed-phase chromatography. These fractions represent a peptide library containing bioactive, desalted and lyophilised peptides of blood. More than 20,000 substances have been identified by MALDI-TOF MS [26]. Thus, this peptide library represents an effective approach for the discovery of potential new

targets for drug development as well as for possible lead structures for drug candidates.

The system set described herein is an improvement on the previously described fast 2D-HPLC system [27]. The implementation of novel approaches and system components significantly improved resolution, speed, reproducibility and robustness for the analysis of peptides of a molecular weight lower than 20 kDa.

2. Experimental

2.1. Hemofiltration

Human hemofiltrate is obtained from the Nephrologisches Zentrum Niedersachsen, Hannoversch-Münden, Germany, in quantities of 1600 l per week. Patients with chronic renal failure are subjected to routine arterio-venous hemofiltration three times per week. The hemofiltration equipment used routinely is a hemoprozessor (Sartorius, Göttingen, Germany) equipped with hemofilters Hemoflow F 60S and Hemoflow HF 80S (Fresenius, Bad Homburg, Germany). All ultrafilters used have a specified molecular mass cut-off around 20 kDa and consist of polysulphone membranes. The effective membrane surfaces are 1.3 m^2 or 1.8 m^2, respectively. The filtration is driven by a transmembranous pressure gradient of 60–100 mmHg at a blood flow-rate of 250–350 ml/min. Twenty to 30 l of filtrate are recovered per patient and treatment.

2.2. Peptide extraction from hemofiltrate

The sterile filtrate is immediately cooled to 4 °C and acidified to pH 3 to prevent bacterial growth and proteolysis. Extraction of the peptides from HF is performed using a strong cation exchanger (Fractogel TSK SP 650(M), Merck, Darmstadt, Germany; 25 cm × 10 cm, Vantage VA 250 column, Amicon, Witten, Germany). The peptide extraction procedure is performed as described in detail by Schulz-Knappe et al. [23]. Briefly, batches of 800 l HF are diluted with water to a conductivity of 5 mS and are conditioned to pH 2.5 with hydrochloric acid. Then, the conditioned filtrate is applied onto the strong cation exchanger using an Autopilot chromatography system (Applied Biosystems, Weiterstadt, Germany). After washing with a volume of 100 l water (pH adjusted to 2.5 with HCl), a batch elution is performed with 7.5 l 0.5 M ammonium acetate (pH 7, three column volumes). The eluate is stored at −20 °C and subsequently lyophilised for further processing.

2.3. Size-selective sample fractionation using novel restricted access columns

A novel restricted access material was applied as column packing (research products of Merck KGaA). Strong cation-exchange restricted access material (SCX-RAM) comprises of spherical LiChrospher 60 (dp: 25 μm) silica-based beds. The outer surface is modified exclusively with electroneutral diol-groups whereas the inner particle surface carries anionic (sulfonic acid) functional groups. The average pore size of the SCX-RAM material is 6 nm corresponding to a molecular cut-off of approximately 20 kDa and the column dimensions are 25 mm × 4 mm i.d.

3. 2D-HPLC system

3.1. Sample fractionation step using SCX-RAM columns

The buffers for the SCX-RAM column and the analytical cation exchanger were 10 mM phosphate buffer at pH 3.0 (buffer A) and 1 M phosphate buffer at pH 3.0 (buffer B).

The SCX-RAM column was loaded with 100 μl (50 μg protein/μl) human hemofiltrate extract processed as mentioned above at a flow rate of 0.2 ml/min applying 100% buffer A. The column was washed for 12 min. Elution of the retained fraction from the SCX-RAM column was performed in a backflush mode in-line with the analytical column using shallow gradient to 100% eluent B at a flow rate of 0.5 ml/min.

3.2. First dimension

The analytical ion-exchange column was TSKgel SP-NPR providing SO_3H functionality. The columns were based on non-porous 2.5 μm polymeric beads, packed in a 35 mm × 4.6 mm i.d. column (TosoHaas, Stuttgart, Germany). The column flow rate was 0.5 ml/min and the column was always operated in-line with the ion-exchange restricted access column.

The linear gradient in the first dimension rose from 10 mM phosphate buffer to 1.0 M phosphate buffer in 96 min. Afterwards, column washing was performed with 1.0 M phosphate buffer in 8 min, followed by 8 min column regeneration using buffer A prior to the next 2D-analysis.

3.3. Second dimension

The four similar reversed-phase columns were MICRA ODS I consisting of non-porous C-18 modified silica beads (1.5 μm) packed in 14 mm × 4.6 mm i.d. columns (Eichrom Technologies Inc., Darien, IL, USA).

The eluents for RP separations were A: 0.1% (v/v) trifluoroacetic acid (TFA) in water and B: 0.1% (v/v) TFA in acetonitrile. The gradient cycle for the reversed-phase columns started with 4% B, which was increased to 40% B within 6 min and further increased to 100% B within 0.66 min, then maintained for 0.15 min. Initial column regeneration and tube flushing was performed at 4% B for 1.17 min using a flow rate of 2 ml/min. Additional, reversed-phase column regeneration prior to new sample enrichment was

performed with water containing 0.1% (v/v) TFA at a flow rate of 0.5 ml/min. All buffers and TFA were of analytical grade (Fluka Chemika, Buchs, Switzerland), while eluents were HPLC gradient grade (Merck). Pure water was obtained using a Milli-Q device (Millipore Bedford, MA, USA). All separations were performed at ambient temperature.

3.4. Operation of the 2D-HPLC system

The column arrangement was fully automated and controlled by an Integral^{TM} 100Q workstation (Applied Biosystems, Framingham, MA, USA). The workstation provides a high-pressure gradient HPLC system comprising a UV detector, refrigerated autosampler and three ten-port valves. Two HPLC gradient systems, a pump and two more ten-port valves were added to build the total system (Fig. 1). A detailed description is given in [9].

Three of the ten-port valves are integrated in the Integral^{TM} 100Q workstation, pneumatically operated and made of PEEK (Rheodyne, Rhonert Park, CA, USA). Two external electrically driven ten-port valves (Valco Instruments Co. Inc., Houston, TX, USA) were controlled by electrical contact closures from the Integral^{TM} 100Q workstation. Two individual high-pressure gradient systems were used for the second dimension. One system consisted of two HPLC pumps model 2200 and a central processor model 7110 for gradient control (Bischoff Analysentechnik, Leonberg, Germany). The second pump set consisted of two LC-10 AD pumps (Shimadzu, Kyoto, Japan). Gradient mixing was performed using two dynamic low void volume mixing chambers (Bischoff Analysentechnik). An isocratic pump (LKB 2150, Pharmacia, Uppsala, Sweden) was used for column regeneration. One UV detector was part of the workstation and equipped with a 1.2 μl flow cell, while the second detector was a Lambda 1000 (Bischoff Analysentechnik) equipped with a 0.8 μl flow cell; both detectors were operated at a wavelength of 215 nm. The samples were stored in 1.2 ml vials at 3 °C in the temperature-controlled autosampler tray. Injections were automatically performed by filling a 100 μl sample loop.

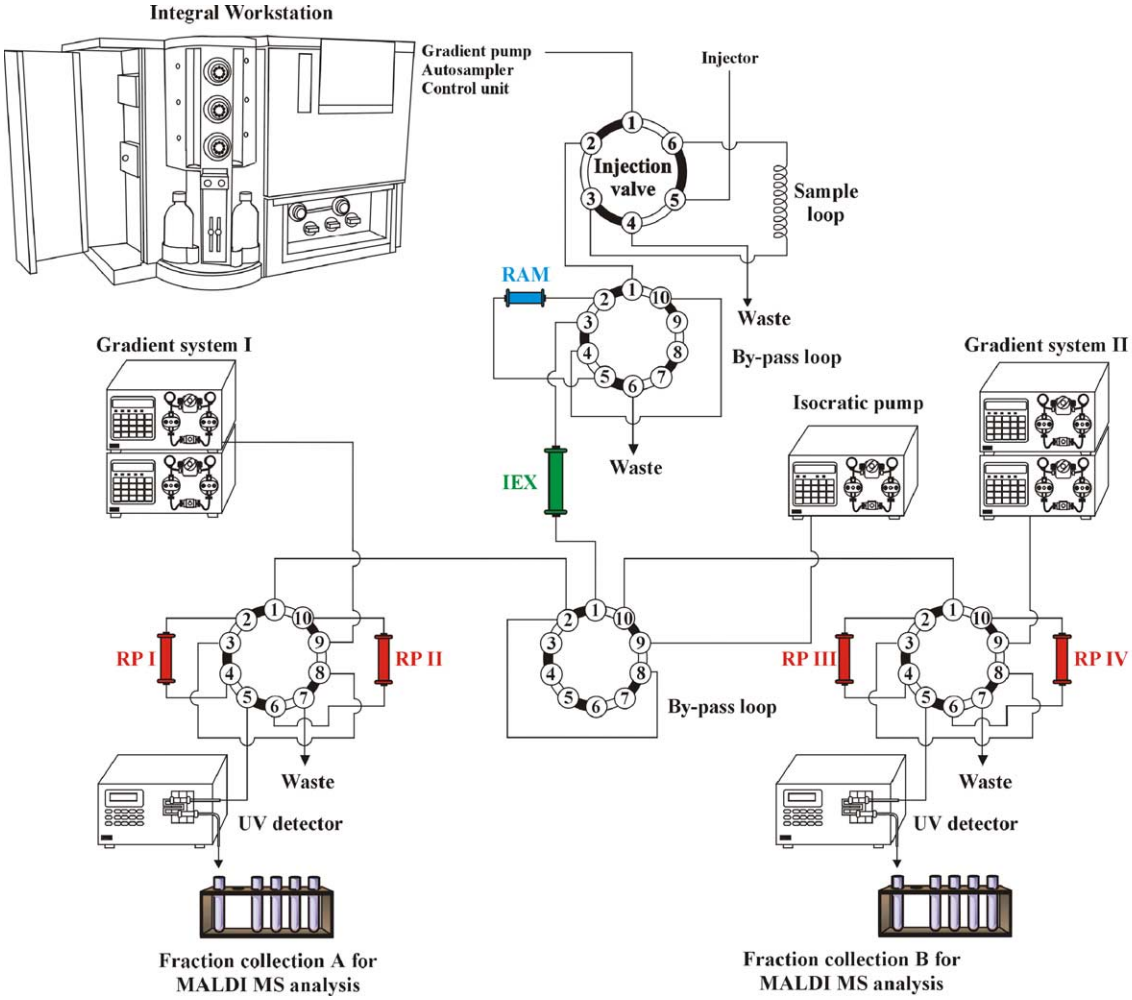

Fig. 1. Schematic diagram of the on-line comprehensive two-dimensional HPLC system including an integrated sample preparation step.

3.5. MALDI-TOF MS

MALDI mass spectra were obtained on a Voyager DE Pro mass spectrometer (Applied Biosystems, Weiterstadt, Germany) equipped with a nitrogen laser used at 337 nm. The accelerating voltage in the ion source was set to 25 kV. Data were acquired in the positive linear operation mode using a 1.2 m flight tube. Time-to-mass conversion was achieved by external calibration using standards of bradykinin, human secretin, and human ubiquitin obtained from Sigma Chemical Co. (St. Louis, MO, USA). All experiments were performed using a matrix of α-cyano-4-hydroxycinnamic acid mixed with L-fucose (both Sigma–Aldrich, Steinheim, Germany) dissolved in a 50% (v/v) mixture of ACN/0.1% aqueous TFA (2.5 mg/ml each). Equal volumes of matrix and sample solutions (1 μl) were mixed on a stainless steel multiple sample tray according to the dried drop technique. Data acquisition and analyses were performed using the Voyager version 5.10 and the Data Explorer 4.0.0.0 software supplied by the manufacturer. For improved accuracy (±0.3%), MALDI-TOF MS measurements were repeated after the addition of an internal peptide standard for mass calibration (average MW: 3913.4).

3.6. Amino acid sequence analysis

N-terminal sequencing was performed on a 473A gas phase-sequencer and a Procise 494 sequencer (Applied Biosystems, Weiterstadt, Germany) by Edman degradation with on-line detection of phenylthiohydantoin-amino acids using the standard protocol recommended by the manufacturer.

4. Results and discussion

4.1. Multi-dimensional system approach

The basic idea of an on-line comprehensive 2D-HPLC system is the use of an on-line sample clean-up strategy using two directly connected separation dimensions. In the first dimension, a strong cation-exchange restricted access media column is followed by an analytical cation-exchange column. In the second dimension, four parallel reversed-phase columns enable a fast separation [9] as shown in Fig. 1. Enrichment of the fractions directly on top of the column does not require any sample storage, hence there is no vial contamination/wall adsorption or sample loss due to other sample handling procedures such as fraction collection and reinjection. The procedure avoids sample dilutions and automatically desalts the analytes, thus preventing eluent incompatibilities. The system recoveries and reproducibility was proved for standard peptides [9].

Fig. 2 shows the analytical cation-exchange separation of a human hemofiltrate sample at pH 3.0. After loading, the cation-exchange RAM-column was eluted in-line coupled to the analytical cation-exchange column. The chromatogram demonstrates a separation of the molecular weight fraction below 20 kDa within the 96 min analysis time. Approximately 25 peaks were visible but not baseline resolved.

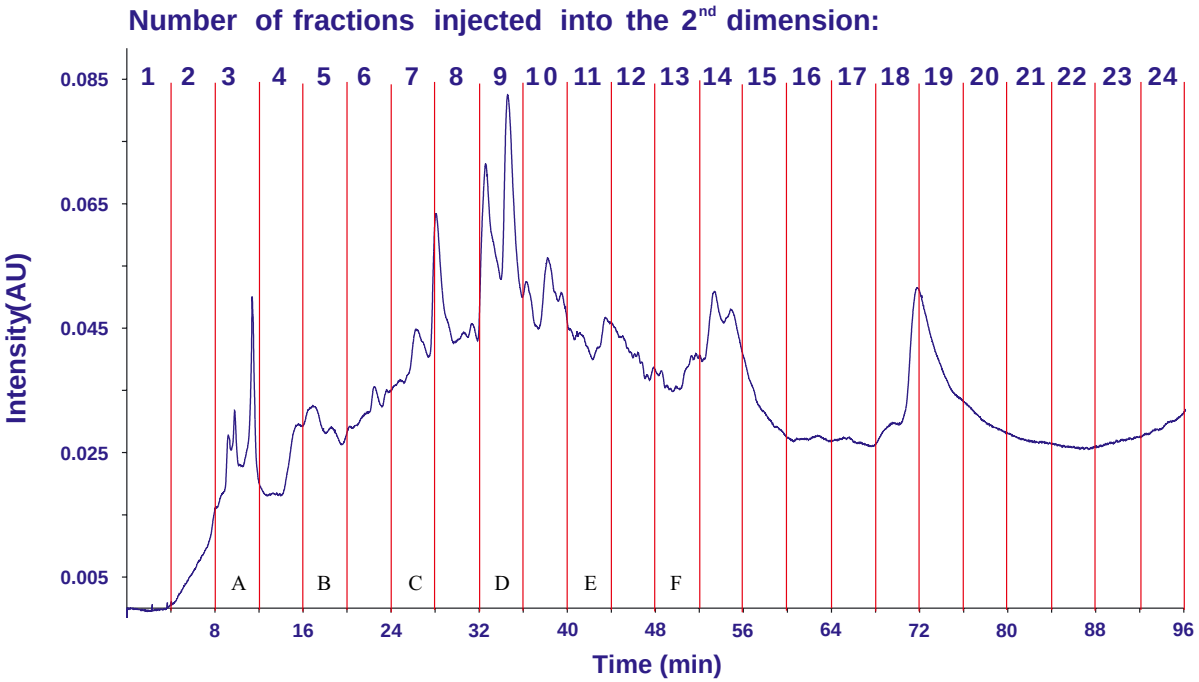

Fig. 2. The chromatogram illustrates the separation of human hemofiltrate on the analytical cation-exchange column in the first dimension after first being subjected to selective enrichment on a cationic RAM. Fractions (24 in total) were continually transferred to the second dimension in 4 min intervals for subsequent analysis by reversed-phase chromatography.

Each of the 24 fractions of 4 min duration (2 ml eluent) in Fig. 2 were transferred on-line to the second dimension for subsequent reversed-phase chromatography on MICRA ODS I columns. These 24 fractions (Fig. 2) were separated simultaneously on the four reversed-phase columns resulting in six reversed-phase chromatograms for each RP column. Fig. 3 shows exemplarily six typical reversed-phase chromatograms (Fig. 3A–F) generated during a complete two-dimensional (SCX-RAM/SCX/RP) run.

Automatic fractionation of equidistant intervals, regardless of the first dimension separation pattern, was applied for sample transfer. A total number of 24 RP-chromatograms were generated in the second dimension with more than 60 peaks resolved in same chromatograms with low

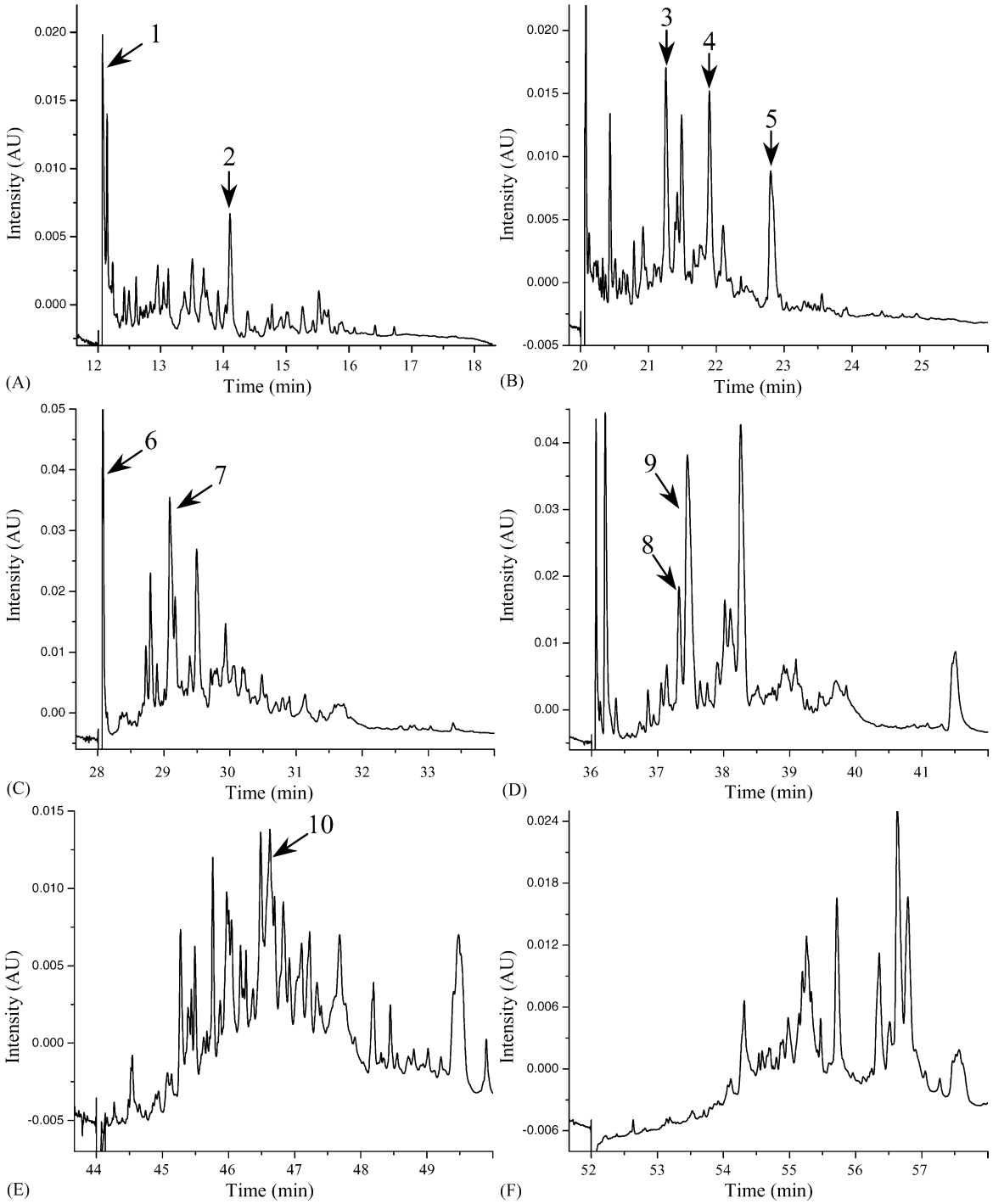

Fig. 3. Selected reversed-phase chromatograms (corresponding to fractions A–F marked in Fig. 2) showing a complete two-dimensional RAM/cation-exchange/reversed-phase run exemplifying the high resolving power within 8 min analysis time. Numbered and marked peak fractions 1–10 were selected for MS and sequence analysis (Table 1, Fig. 4).

overlapping that confirms the orthogonal separation power. More than 1000 peaks were resolved within the total analysis time of 96 min.

Despite a total number of 1000 resolved peaks in the 2D-system, it does not necessarily signify that 1000 different components are resolved, as would be expected in a single mode run. Usually the same component could be found in several consecutive MS spectra. This is an intrinsic feature of the column coupling approach.

It was demonstrated that high-resolution power with minimal sample preparation can be achieved at a concentration level which is accessible by UV detection. This offers the potential to map expression differences (up and down regulations) by comparing the UV traces. As demonstrated by mass determination (Fig. 4), the HPLC fractions are of low complexity indicating the excellent chromatographic resolving power.

4.2. Peptide identification approach

The unique importance of proteins and peptides as hormonal substances for the organised function of cells and whole organisms is reflected in all complex biological systems. The blood plasma contains most of the secreted peptides and is therefore the most comprehensive source for the systematic identification of regulatory peptides present in the human organism. Since regulatory peptides occur in very low concentrations, large amounts of starting material are necessary for their isolation. Therefore, the use of whole blood or plasma is very limited. Alternatively, human hemofiltrate obtained from patients with chronic renal failure can be used as a source for the isolation of circulating peptides and is available in large quantities. Fractionation of the complex peptide mixture in hemofiltrate via cation-exchange chromatography followed by analytical HPLC and mass spectrometry allowed the detection of a high number of peptides (∼1500) [23].

Due to the molecular weight cut-off of the hemofilters (20 kDa), plasma proteins are excluded to such an extent that the total protein content is only 50–70 mg/l compared to 70 g/l in plasma.

As shown by Schepky et al. [24], plasma and HF concentrations of small hormones such as angiotensin II, vasopressin, gastrin, endothelin and insulin are in the same range whereas only 0.02% of blood albumin is passed through the hemofilter [24]. This filtration is useful for the enrichment of smaller peptides during purification procedures and enables to isolate peptides from 10,000 l of plasma equivalent.

Our study aimed to prove that the multi-dimensional system presented could map and separate peptides with high-resolution to enable the analysis of single peaks corresponding to single fractions and to allow the identification of the pertinent peptides by mass spectrometry and Edman degradation. Therefore, several single major peak fractions detected by UV absorbance in the RP chromatography were collected as shown in Fig. 3. Proteins were identified by a

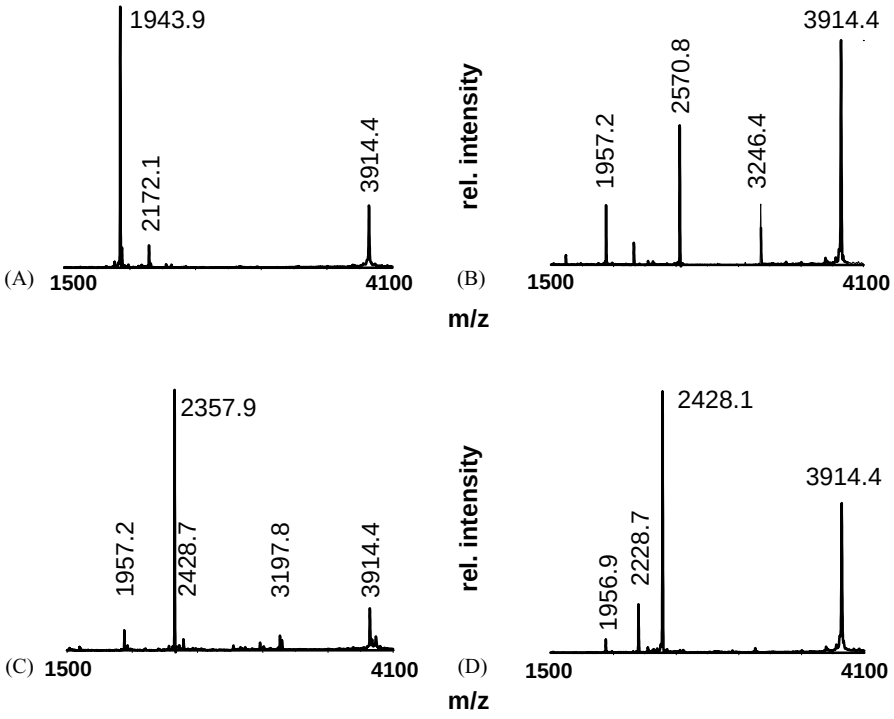

Fig. 4. MALDI-TOF mass spectra of selected RP-HPLC fractions (A) 4, (B) 7, (C) 8 and (D) 9 corresponding to numbered peaks of Fig. 3. *m/z* 3914.4 (doubly charged: 1957.2) is an internal peptidic standard for mass calibration. Measurement was performed in the linear positive operation mode using a matrix of α-cyano-4-hydroxycinnamic acid mixed with fucose. Assigned peptides were identified by Edman sequencing as shown in Table 1.

Table 1
Identified peptides from human hemofiltrate after 2D-LC separation

Fraction number	Determined mass (Da)[a]	Determined sequence[b]	Identified compound	Theoretical mass (Da)	AC number[c]	Known physiological function of the entire protein
1	1387.1	EEVSGNVSPGTRR	Fibrinogen α/α-E precursor 414–426	1387.5	P02671	Polymerisation into fibrin and acting as a cofactor in platelet aggregation
	1516.4	EEVSGNVSPGTRRE	Fibrinogen (α/α-E precursor 414–427	1516.6	P02671	
2	2506.6	LMIEQNTKSPLFMGKVVNPTOK	α1-Anti-trypsin precursor 397–418	2504.0	P01009	Inhibitor of serine proteinases like elastase, plasmin and thrombin
3	1716.9	VRYTKKVPQVSTPTL	Serum albumin precursor 433–447	1717.0	P02768	Binding of water, Ca^{2+}, Na^{+}, K^{+}, fatty acids, hormones, and bilirubin; regulation of colloidal osmotic pressure
4	1943.9	LLVRYTKKVPQVSTPTL	Serum albumin precursor 431–447	1943.4	P02768	See above
	2172.1	LLVRYTKKVPQVSTPTLVE	Serum albumin precursor 431–449	2171.6	P02768	See above
5	3055.0	SSPGKPPRLVGGPMDASVEEEG	Cystatin C precursor 27–55	3054.4	P01034	Inhibitor of cysteine proteinases
6	1406.8	AKIRDRSTSGGKM	Insulin-like growth factor BP-4 144–156	1406.6	P22692	Half-life prolongation of IGFs and alteration of cell surface receptor interaction
	2422.5	MADEAGSEADHEGThSTKRGH	Fibrinogen α/α-E precursor 603–625	2422.5	P02671	See above
7	3246.4	EEVSGNVSPGTRREYHTEKLVT	Fibrinogen α/α-E precursor 414–442	3246.5	P02671	See above
	2570.8	IQRTPKIQVYSRHPAENGHSNF	β2-Microglobulin precursor 21–42	2570.9	P01884	β-Chain of major histocompatibility complex class I molecules
8	2357.9	DAHKSEVAHRFKDLGEENFK	Serum albumin precursor 25–44	2357.2	P02768	See above
	2428.7	DAHKSEVAHRFKDLGEENFKA	Serum albumin precursor 25–45	2428.7	P02768	See above
	3197.8	SARGHRPLDKKREEAPSLRPAPP	Fibrinogen β-chain precursor 42–71	3197.6	PO2675	See above
9	2228.7	DAHKSEVAHRFKDLGEENF	Serum albumin precursor 25–43	2229.4	P02768	See above
	2428.1	DAHKSEVAHRFKDLGEENFKA	Serum albumin precursor 25–45	2428.7	P02768	See above
10	2753.8	DAHKSEVAHRFKDLGEENFKA	Serum albumin precursor 25–48	2754.1	P02768	See above
	2864.8	LLKNGERIEKVEHSDLSFSKDW	β2-Microglobulin precursor 59–82	2865.2	P01884	See above
	3856.5	AVGEYNKASNDMYHSRALOVV	Cystatin C precursor 56–89	3856.4	P01034	See above

[a] Masses obtained using MALDI-TOF MS as $[M + H]^{+}$.
[b] Sequence determined by Edman degradation.
[c] Accession number of the Swiss-Prot database.

combination of MALDI-TOF MS and Edman degradation. Eight different precursor proteins were identified (Table 1). In average, two components were found per fraction showing high-resolution of the multi-dimensional system also enabling the identification by MS. The demonstrated high-resolution capacity offers the option to successfully load and separate even more complex mixtures of peptides. Contrary to 2D-chromatography of peptide fractions, 2D-gel plasma maps [8] predominantly detect proteins $M_r > 10$ kDa. The smallest proteins found in this 2D-gel map were kininogen light chain and apolipoprotein A-II with an $M_r = \sim 10$ kDa. This shows that our 2D-chromatography approach in which 95% of the detected substances are smaller than 3.5 kDa yields information complementary to a 2D-gel plasma map. For peptide identification, N-terminal sequence analysis of about 10 amino acids was carried out using conventional Edman chemistry. This N-terminus was then compared to data bases available. When a known peptide was identified, its molecular mass was calculated from the extended amino acid sequence obtained from the data base and was compared to the measured mass (Table 1).

The observed detection limit of identified different peptides was approximately 1 pmol/l as calculated by integration of the total peak area and comparison to the single collected fraction area taking in account that the RAM column reduces the mass load onto the system. This suggested minimal concentrations for the detection of these molecules in a range from 3 to 300 pM, but detection of a peptide by 2D-chromatography is dependent on the concomitant components in the analyte, which may dramatically increase the detection limit for such peptides.

The presented 2D-separation technology may accelerate the completion of a peptide database based on mass and sequence [26].

This database of circulating human peptides contains over 1000 entries from about 75 different protein and peptide precursors (e.g. from plasma fibrinogen or albumin, complement factors, enzymes, enzyme inhibitors and transport proteins); 4% of the entries are attributable to peptide hormones and 3% to growth factors and cytokines [26].

Due to the progress in genome sequencing, most of the human proteins are found in common available databases (e.g. in the Swiss-Prot or Expressed Sequence Tags database, dbEST), but their post-translational processing has to be elucidated carefully by the analysis of the naturally occurring (poly-)peptides. Various endogenous proteolytic products of large proteins are separated and detected by the herein described technique as demonstrated for the high abundant albumin or fibrinogen (Table 1). The identification and mapping of proteolytic cleavage products helps to clarify physiological processes and in vivo degradations. Furthermore, peptidic products from those proteins might possess individual biological activity as shown for the anti-angiogenic endostatin [28,29], for the anti-microbial LEAP-2 [30] or for the serine proteinase inhibiting LEKTI [31]. Therefore, our automated 2D-HPLC technology may contribute to the analysis of the peptidome of organisms and could support the identification of peptidic disease markers.

5. Conclusions and perspectives

The resolution power of the 2D-HPLC set-up is superior to any other separation method for the specific molecular weight range of peptides <20 kDa. 2D-chromatography is a good complement to a 2D-gel plasma map by predominant detection of peptides. This has been clearly demonstrated by the high-resolution separation and identification of peptides below 20 kDa from human hemofiltrate. Hemofiltrate is one of the most complex natural peptide samples known, similar to blood plasma, but only with approximately 1000-fold lower protein content. High-resolution separations were obtained in single peaks from reversed-phase columns which were composed of few components and, thus, similar or even better results are expected for less complex samples. RAM columns are ideally suited for sample preparation of small-sized biopolymers, since they enable to boost the sensitivity for low abundant sample components by loading high amounts of sample. In addition, the higher molecular weight fraction can be excluded by performing direct untreated biological sample injections. High sensitivity was obtained for the 2D-LC system with the ability to identify single components in a concentration below 1 pmol/l.

The new 2D-HPLC method off-line connected to MALDI-TOF MS can easily be used in high-throughput mapping applications of peptides or as a tool for differential peptide display for the discovery of biomarkers from patients or healthy donors.

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